

Sox17 Dependence Distinguishes the Transcriptional Regulation of Fetal from Adult Hematopoietic Stem Cells

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SUMMARY

Fetal stem cells differ phenotypically and functionally from adult stem cells in diverse tissues. However, little is known about how these differences are regulated. To address this we compared the gene expression profiles of fetal versus adult hematopoietic stem cells (HSCs) and discovered that the Sox17 transcriptional regulator is specifically expressed in fetal and neonatal but not adult HSCs. Germline deletion of Sox17 led to severe fetal hematopoietic defects, including a lack of detectable definitive HSCs. Conditional deletion of Sox17 from hematopoietic cells led to the loss of fetal and neonatal but not adult HSCs. HSCs stopped expressing Sox17 approximately 4 weeks after birth. During this transition, loss of Sox17 expression correlated with slower proliferation and the acquisition of an adult phenotype by individual HSCs. Sox17 is thus required for the maintenance of fetal and neonatal HSCs and distinguishes their transcriptional regulation from adult HSCs.

INTRODUCTION

Stem cells undergo discrete developmental changes throughout life but little is known about how these transitions are regulated. A particularly profound transition occurs between fetal development and adulthood, when stem cells from diverse tissues undergo changes in phenotype, function, and regulation (Molofsky et al., 2004). This raises the possibility that transcriptional programs that maintain stem cell identity and function change between fetal and adult life.

One cardinal feature of stem cells that changes with development is self renewal. Even self-renewal mechanisms that are broadly conserved among tissues fail to be maintained across developmental time (Molofsky et al., 2004).

Oct4 and *Nanog* are required for the self renewal of embryonic stem cells but not fetal or adult somatic stem cells (Nichols et al., 1998; Chambers et al., 2003; Mitsui et al., 2003). *Bmi-1* is required for the self renewal of every post-natal stem cell yet examined, including HSCs and neural stem cells, but it is not required in vivo for the self renewal of fetal stem cells in the same tissues (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003). *Ink4a* expression cannot be detected in fetal or young adult stem cells but increases with age in stem cells from diverse tissues, reducing self renewal potential and regenerative capacity (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). While a great deal has been learned about embryonic and adult stem cell self renewal, comparatively less is known about mechanisms that specifically maintain fetal stem cells.

Developmental changes in the properties of stem cells have been best described in hematopoiesis (Mikkola and Orkin, 2006). Fetal HSCs differ from adult HSCs in gene expression (Phillips et al., 2000; Ivanova et al., 2002), marker expression (Morrison et al., 1995; Kim et al., 2005), developmental potential (Ikuta et al., 1990; Kantor et al., 1992), self-renewal potential (Morrison et al., 1995; Harrison et al., 1997), and regulation (Park et al., 2003; Hock et al., 2004a, 2004b). HSCs transition from fetal to adult properties 3–4 weeks after birth, when HSCs suddenly become quiescent (Bowie et al., 2006).

It is not clear what regulates the unique properties of fetal HSCs. A number of genes including *Scf* (Porcher et al., 1996), *Aml-1/Runx1* (Okuda et al., 1996; Wang et al., 1996), *Lmo2* (Warren et al., 1994), *Gata-2* (Tsai et al., 1994), *cdx4* (Davidson et al., 2003), and *mixed-lineage leukemia* (Ernst et al., 2004) are required embryonically for the formation of HSCs. However, with the exception of *Gata-2*, there is little evidence that these genes play any ongoing role in the maintenance of HSCs after formation (Mikkola and Orkin, 2006). For example, *Scf* and *Aml-1/Runx1* are dispensable for the maintenance of HSCs, at least in adulthood (Mikkola et al., 2003; Ichikawa et al., 2004). Other genes appear to regulate the maintenance of HSCs throughout fetal and adult life including *Rae28* (Ohta et al., 2002; Kim et al., 2004), *Meis1* (Hisa et al.,

2004; Kirito et al., 2004; Azcoitia et al., 2005), *c-myb* (Mucenski et al., 1991; Sandberg et al., 2005), and *Cbp* (Rebel et al., 2002). However, to our knowledge no gene is known to regulate the maintenance of fetal but not adult HSCs. In contrast, a number of transcriptional regulators maintain adult but not fetal HSCs, including Gfi-1 (Hock et al., 2004a), Tel/Etv6 (Hock et al., 2004b), and Bmi-1 (Park et al., 2003). This raises the question of what transcriptional regulators act downstream of Scl and Aml-1/Runx1 to regulate fetal HSC identity and maintenance prior to the onset of the adult HSC self-renewal program.

Sry-related high mobility group box (Sox) transcription factors contain a DNA-binding domain (the HMG box) and regulate stem cell identity and function in multiple tissues (Schepers et al., 2002). *Sox17* has been used as a marker of endodermal identity (Yasunaga et al., 2005) and is required for the formation and maintenance of endoderm (Hudson et al., 1997; Kanai-Azuma et al., 2002) and vascular endothelium (Matsui et al., 2006). But Sox family members have never been implicated in the regulation of HSCs. Here we report that within the hematopoietic system *Sox17* is highly restricted in its expression to fetal and neonatal HSCs and is required for the maintenance of fetal and neonatal, but not adult HSCs. *Sox17* is thus a marker of fetal identity in HSCs and distinguishes their transcriptional regulation from adult HSCs.

RESULTS

In this study we reexamined previously published gene expression profiles of highly purified fetal (Kiel et al., 2005a) and adult (Kiel et al., 2005b) mouse HSCs to identify genes that were more highly expressed in fetal as compared to adult HSCs. *Sox17* was clearly detected in embryonic day (E)14.5 fetal liver HSCs, but not in CD45⁺ fetal liver hematopoietic cells, young adult bone marrow HSCs, young adult bone marrow CD45⁺ cells, or old adult bone marrow HSCs (Figure S1). We confirmed this by quantitative real-time RT-PCR (Figure S1). We hypothesized that *Sox17* regulates fetal HSC identity or function.

Generation of *Sox17*-Deficient Mice

To test whether *Sox17* regulates fetal HSCs we generated *Sox17* knockout mice. The entire coding sequence of *Sox17* was replaced with the gene encoding enhanced green fluorescence protein (eGFP), in frame with the *Sox17* start codon, by homologous recombination (Figures 1A and 1B). Two mouse lines were generated using independently targeted ES cell lines, both of which gave germline transmission of *Sox17*^{GFP} alleles. We could not detect mRNA that contained the *Sox17* coding sequence in *Sox17*^{GFP/GFP} mice by RT-PCR (Figure 1C). *Sox17*^{GFP/GFP} mice from both lines showed severe growth retardation relative to littermate controls by E11.5, as well as posterior patterning and body axis rotation defects (Figure 1D). In both lines, *Sox17*^{GFP/GFP} mice were observed in expected ratios through E12.5 but died by E13.5 (Figure 1E). This phenotype is consistent with that

of independently targeted *Sox17*-deficient mice that also died embryonically with growth retardation and posterior patterning defects (Kanai-Azuma et al., 2002).

Sox17 Deficiency Severely Reduces Fetal Hematopoiesis

In addition to recapitulating the previously described gross phenotypes associated with *Sox17* deficiency, we observed a severe defect in fetal hematopoiesis that was not reported previously. *Sox17*^{GFP/GFP} mice had some blood cells and hemorrhaging but did not develop visible hematopoiesis as observed in control littermates (Figures 1D and 2B). This failure of hematopoiesis affected the yolk sac (Figure 2A) as well as the fetal liver (Figure 2B) as both structures were pale in *Sox17*^{GFP/GFP} mice. *Sox17*^{GFP/GFP} mice had significantly ($p < 0.05$) fewer CD45⁺ blood cells and Ter119⁺ erythroid cells in both the yolk sac and whole embryo as compared to littermate controls (Figure 2C). *Sox17*^{GFP/GFP} mice also exhibited significantly fewer colony-forming progenitors in yolk sac and whole embryo (CFU-C) (Figure 2D). All types of CFU-C were similarly reduced in number (data not shown). The fact that these defects were most severe intraembryonically suggests that *Sox17* is particularly important for definitive hematopoiesis.

In the Hematopoietic System *Sox17* Is Mainly Expressed by Fetal and Neonatal HSCs

To determine how broadly *Sox17* is expressed we examined GFP fluorescence in *Sox17*^{GFP/+} mice. Only 0.5% of E14.5 fetal liver cells were GFP⁺, and this percentage declined over time, with only 0.2% GFP⁺ cells in bone marrow from 2-week-old mice and levels similar to background in bone marrow from 4- and 8-week-old mice (Figure 3A).

To examine GFP expression in fetal HSCs we examined Sca-1⁺Lineage[−]Mac-1⁺CD48[−] cells from E14.5 fetal liver, which represented $0.030 \pm 0.006\%$ of fetal liver cells in these experiments. These cells include all fetal liver HSC activity and are highly enriched for HSCs (Kim et al., 2006). Ninety-four percent (two percent standard deviation) of Sca-1⁺Lineage[−]Mac-1⁺CD48[−] cells were GFP⁺ in *Sox17*^{GFP/+} mice (Figure 3B). To functionally test whether all E14.5 fetal liver HSCs express *Sox17*, we transplanted 1,000 GFP⁺ fetal liver cells or 199,000 GFP[−] fetal liver cells from *Sox17*^{GFP/+} mice into irradiated recipient mice in a competitive reconstitution assay. All 12 of the mice transplanted with GFP⁺ cells showed long-term multilineage reconstitution by donor cells, while none of 11 mice transplanted with GFP[−] cells showed any donor cell reconstitution (Figure 3C). These data demonstrate that all, or virtually all, fetal liver HSCs express *Sox17*.

To examine GFP expression in postnatal bone marrow HSCs, we examined Sca-1⁺Lineage[−]c-kit⁺CD48[−] cells from the bone marrow of 2-, 4-, and 8 week-old mice. These cells represented $0.019\% \pm 0.004\%$ of bone marrow cells at 2 weeks but fell to $0.004\% \pm 0.0004\%$ of bone marrow cells by 8 weeks of age. This population contains

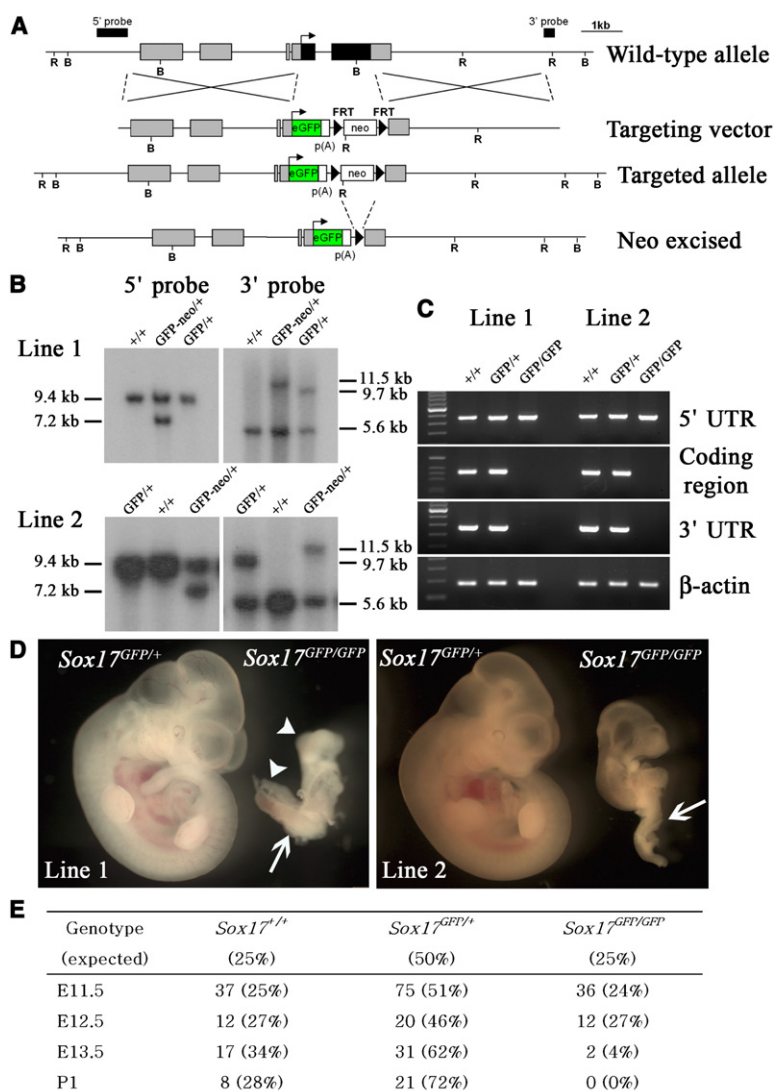


Figure 1. Generation of *Sox17^{GFP/GFP}* Mice

(A) Schematic representation of the *Sox17* targeted allele. The genomic structure after neo-cassette removal is shown on the fourth line. B and R indicate *Bam*HI and *Eco*RV digestion sites, respectively.

(B) Genotypes of heterozygous F1 mice (before neo excision) and F2 mice (after neo excision) from two independent lines were confirmed by Southern blot. Tail genomic DNA was digested with *Eco*RV and *Bam*HI for 5' and 3' probe hybridization, respectively.

(C) The lack of *Sox17* expression in *Sox17^{GFP/GFP}* embryos from both lines of mice was confirmed by RT-PCR using primers that amplified the *Sox17* 5' untranslated region (which was expressed from the targeted allele), the coding sequence (not expressed), and the *Sox17* 3' untranslated region (not expressed).

(D) At E11.5, *Sox17^{GFP/GFP}* embryos were much smaller than *Sox17^{GFP/+}* embryos and exhibited severe defects in posterior patterning (arrow) and body axis rotation (arrowheads).

(E) Genotypes of progeny from *Sox17^{GFP/+}* intercrosses revealed Mendelian inheritance up to E12.5 but loss of *Sox17*-deficient embryos by E13.5.

all of the detectable HSC activity in adult bone marrow and is highly enriched for HSCs (Kiel et al., 2005b; Yilmaz et al., 2005). While $86\% \pm 5\%$ of bone marrow Sca-1⁺Lineage⁻c-kit⁺CD48⁻ cells in 2-week-old mice expressed GFP, this percentage dropped to $50\% \pm 8\%$ in 4-week-old mice and to undetectable levels in 8-week-old mice (Figure 3B). *Sox17* thus continues to be expressed by neonatal, but not adult, HSCs.

Some CFU-C also expressed *Sox17* in the fetal liver. All cells that formed CFU-GEMM colonies (containing granulocytes, erythrocytes, macrophages, and megakaryocytes) in culture arose from GFP⁺ fetal liver cells, as did $65\% \pm 2\%$ of all colony-forming progenitors (CFU-C) from fetal liver (data not shown). By 2 weeks after birth, only $18\% \pm 4\%$ of CFU-GEMM and $9\% \pm 3\%$ of all CFU-C arose from GFP⁺ bone marrow cells. We have not been able to detect GFP⁺ bone marrow cells in adults (Figure 3A). *Sox17* is thus expressed by fetal and neonatal HSCs as well as some downstream progenitors.

To test whether *Sox17* expression is induced in proliferating adult HSCs we treated adult *Sox17^{GFP/+}* mice with cyclophosphamide/G-CSF (Morrison et al., 1997). Although HSCs were driven into cycle, expanded in number, and mobilized after cyclophosphamide/G-CSF treatment, GFP was not expressed by HSCs in the bone marrow or the spleen (Figure S2). This indicates that *Sox17* is not simply expressed by dividing HSCs and cannot be induced in adult HSCs by proliferation or mobilization.

Sox17 Is Required for the Generation or Maintenance of Fetal HSCs

To test whether *Sox17* is required for HSC function during fetal development we generated E12.5 *Sox17^{+/+}*, *Sox17^{GFP/+}*, or *Sox17^{GFP/GFP}* embryos and transplanted yolk sac, fetal liver, or dissociated cells from the rest of the embryo into irradiated recipients in competitive reconstitution assays. Each CD45.1⁺ recipient mouse was

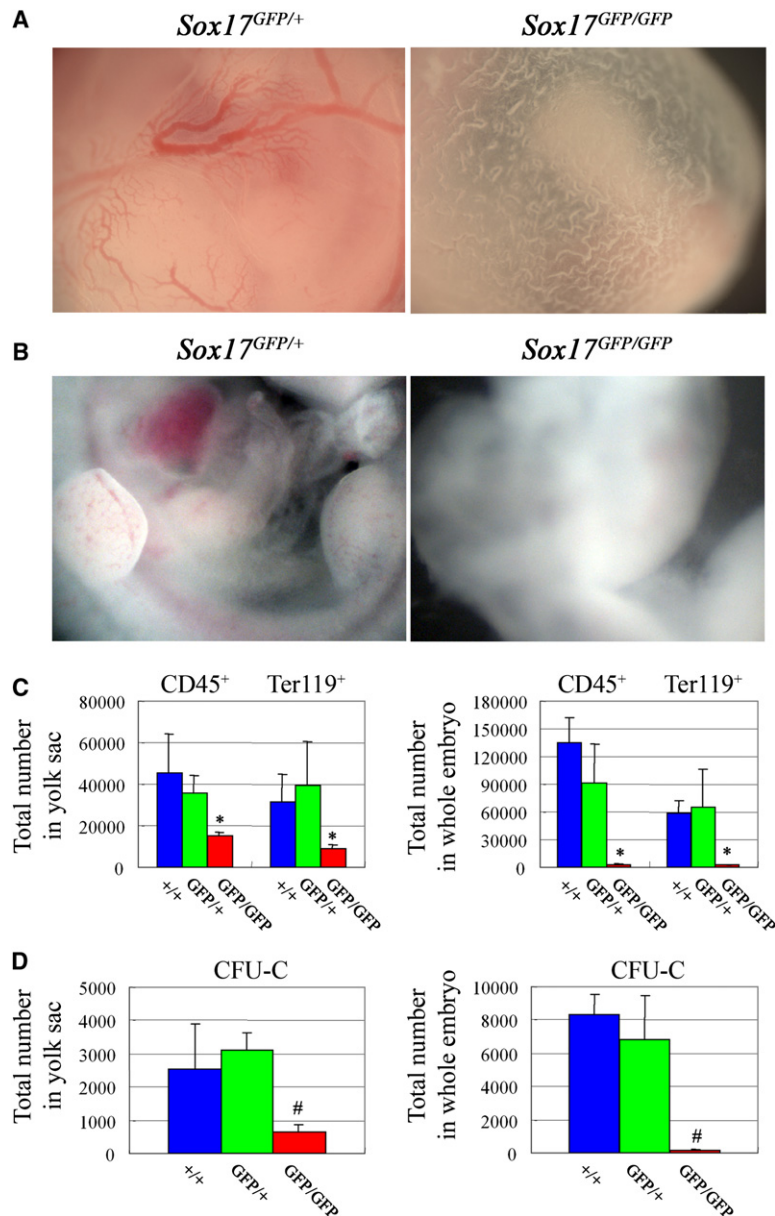


Figure 2. *Sox17* Is Required for Hematopoiesis in the Fetal Liver and Yolk Sac

The yolk sac (A) and fetal liver (B) of E11.5 embryos contained obvious blood cells in *Sox17*^{GFP/+} embryos but not in *Sox17*^{GFP/GFP} littermates. The numbers of CD45⁺ hematopoietic cells and Ter119⁺ erythroid cells in E11.5 yolk sac and whole embryo were significantly (*, $p < 0.05$) reduced in *Sox17*^{GFP/GFP} embryos as compared to *Sox17*^{GFP/+} and *Sox17*^{+/+} embryos (C, three independent experiments). The numbers of colony-forming progenitors (CFU-C) in E11.5 yolk sac and whole embryo were also significantly (#, $p < 0.01$) reduced in *Sox17*^{GFP/GFP} embryos as compared to *Sox17*^{GFP/+} and *Sox17*^{+/+} embryos (D, three independent experiments).

transplanted with all of the yolk sac, liver cells, or remaining embryo cells from a single CD45.2⁺ donor embryo. The livers of *Sox17*^{GFP/GFP} embryos were so hypocellular and pale that the entire embryos were dissociated and transplanted, rather than just fetal liver. Most of the recipients that were transplanted with liver, yolk sac, or other cells from *Sox17*^{+/+} or *Sox17*^{GFP/+} embryos became long-term multilineage reconstituted by donor cells (Figures 3D and 3E). In contrast, none of the recipients transplanted with cells from *Sox17*^{GFP/GFP} embryos ever became reconstituted by donor cells. *Sox17* is thus required for the generation or maintenance of definitive HSCs.

Sox17 Is Required within the Hematopoietic/Endothelial Lineages for HSCs

To test whether *Sox17* is required autonomously for the generation or maintenance of HSCs we generated a floxed allele (*Sox17*^{fl}) for the conditional deletion of *Sox17* (Figures 4A–4C). The *Sox17*^{fl} allele provided normal *Sox17* function prior to recombination as *Sox17*^{fl/GFP} mice (lacking Cre) were born in expected numbers and appeared developmentally normal (Figure 4F). Moreover, intercrosses between *Sox17*^{fl/+} mice led to the birth of *Sox17*^{fl/fl} mice in expected numbers (Figure S3). *Sox17*^{fl/fl} mice were developmentally normal, had normal blood cell counts, and were fertile (Figure S3).

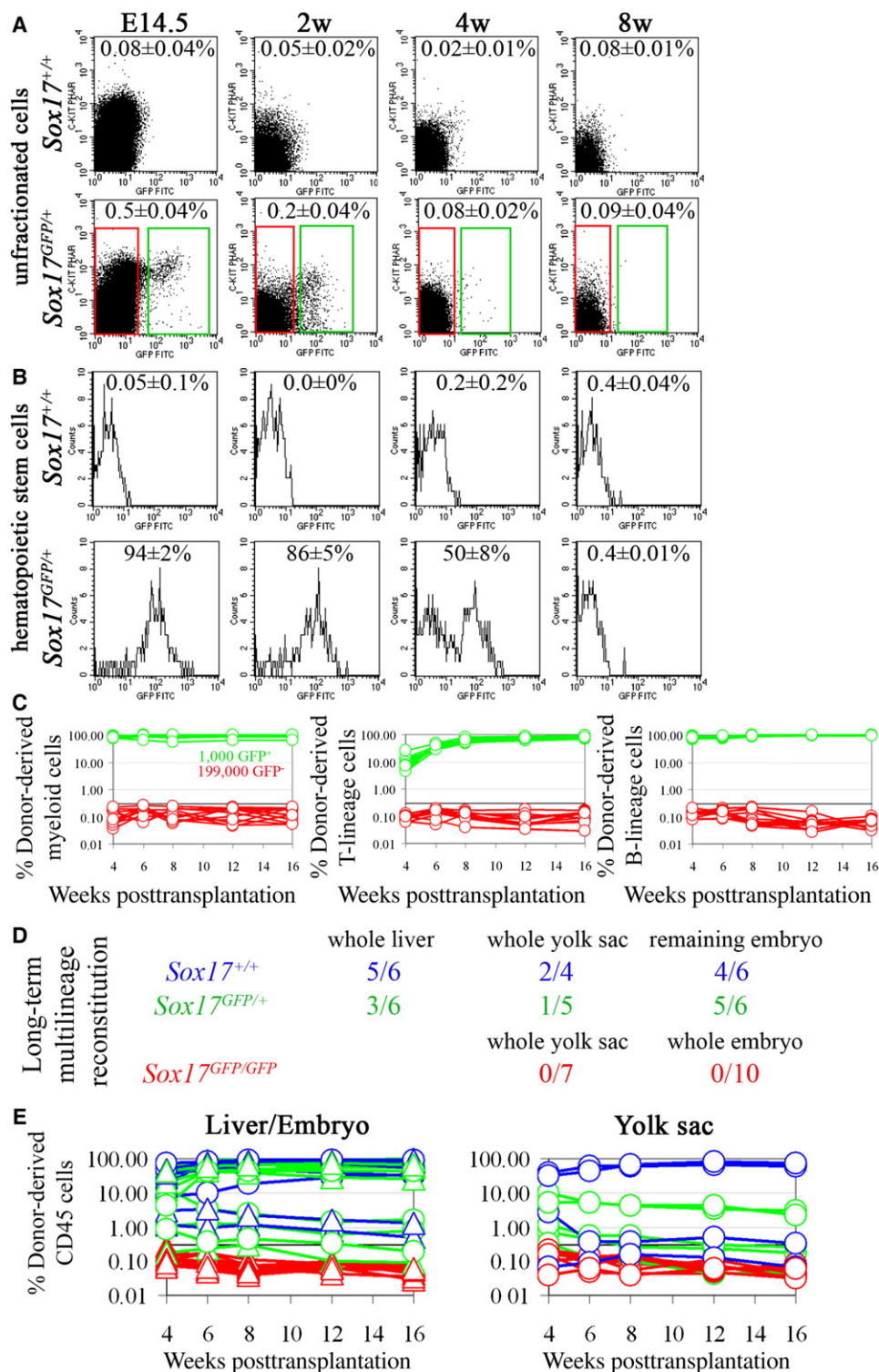


Figure 3. Sox17 Is Expressed by, and Required for the Maintenance of, Fetal and Neonatal But Not Adult HSCs

(A) *Sox17* expression in fetal liver or bone marrow cells was analyzed by flow cytometry in *Sox17^{GFP/+}* mice (five independent experiments). Cells from *Sox17^{+/+}* mice established background. Less than 1% of fetal liver and neonatal bone marrow cells expressed *Sox17* (green boxes).

(B) HSCs were isolated from fetal liver as Sca-1⁺ lineage[−] Mac-1⁺ CD48[−] cells and from bone marrow as Sca-1⁺ lineage[−] c-kit⁺ CD48[−] cells. Most fetal liver and neonatal bone marrow HSCs expressed *Sox17*, but expression began to decline by 4 weeks of age and was no longer evident at 8 weeks of age (five independent experiments).

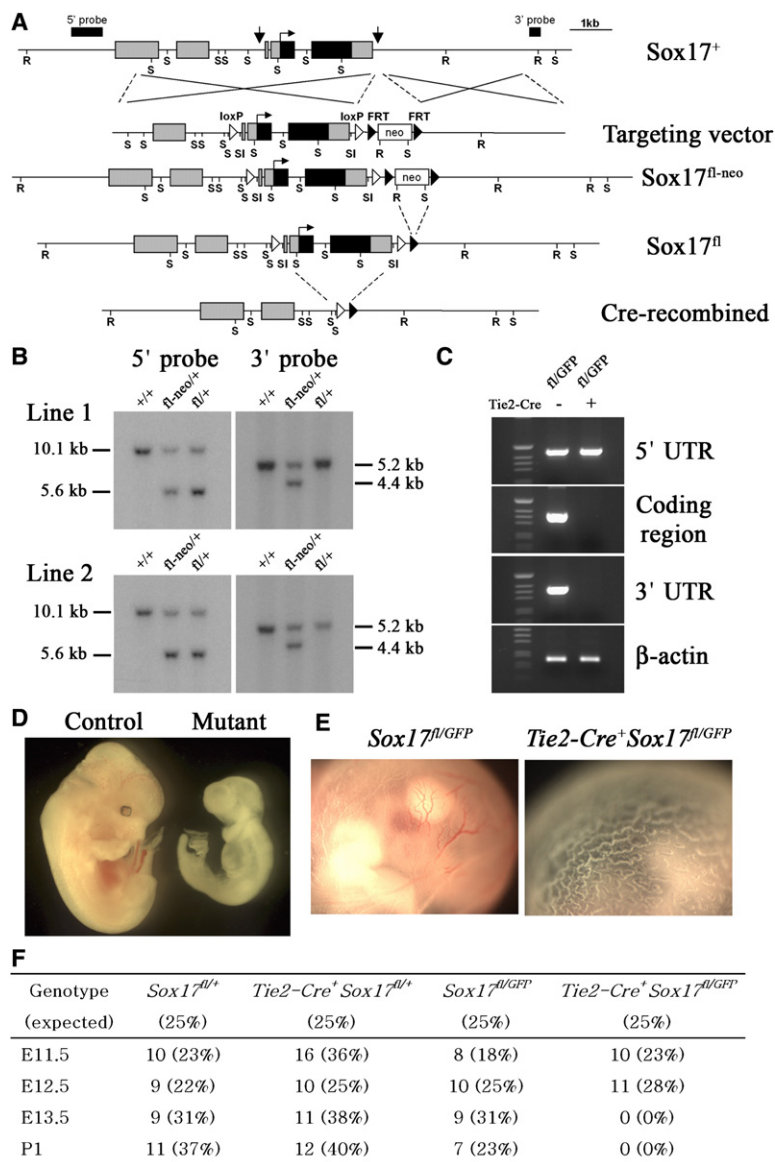


Figure 4. Conditional Deletion of *Sox17* in Hematopoietic/Endothelial Cells Leads to Severe Hematopoietic Defects and Lethality by E13.5

(A) Targeting of *Sox17* to generate a floxed allele. Arrows indicate the sites in the wild-type allele (*Sox17*⁺) where loxP elements were inserted. These sites were selected to avoid disrupting conserved sequences (potential regulatory elements). Note that Cre-mediated recombination removes the entire *Sox17* coding sequence.

(B) The genotypes of heterozygous F1 (*Sox17*^{fl-neo}) and F2 (*Sox17*^{fl}) mice from two independent lines were verified by Southern blot. Genomic tail DNA was digested with *EcoRV* (R) and *SacI* (SI) for the 5' probe and with *SacI* (S) for the 3' probe.

(C) The lack of *Sox17* expression in CD144⁺ endothelial cells sorted from E10.5 *Tie2-Cre*⁺*Sox17*^{fl/GFP} embryos was confirmed by RT-PCR using primers that amplified the *Sox17* 5' untranslated region (expressed from the targeted allele), the coding sequence (not expressed) and the *Sox17* 3' untranslated region (not expressed).

(D) E12.5 *Tie2-Cre*⁺*Sox17*^{fl/GFP} embryos were pale and growth retarded and lacked visible hematopoiesis.

(E) Unlike control embryos, the yolk sac from E12.5 *Tie2-Cre*⁺*Sox17*^{fl/GFP} embryos lacked visible hematopoiesis.

(F) Progeny derived from mating *Tie2-Cre*⁺*Sox17*^{GFP/+} males with *Sox17*^{fl/fl} females: conditional deletion of *Sox17* using *Tie2-Cre* was lethal by E13.5.

To test whether *Sox17* was required within the hematopoietic/endothelial lineages for the generation of HSCs we conditionally deleted *Sox17* in endothelial and hematopoietic progenitors using *Tie2-Cre* (Koni et al., 2001). *Tie2-Cre* mice express Cre recombinase in endothelial cells and HSCs during embryonic development (Schlaeger et al., 2005; Li et al., 2006). E12.5 *Tie2-Cre*⁺*Sox17*^{fl/GFP} embryos were growth retarded and lacked visible hema-

topoiesis in the liver and yolk sac, but did not have the obvious posterior patterning or axial turning defects observed in *Sox17*^{GFP/GFP} embryos (Figures 4D and 4E). Like *Sox17*^{GFP/GFP} embryos, *Tie2-Cre*⁺*Sox17*^{fl/GFP} embryos were observed in expected numbers through E12.5 but were dead by E13.5 (Figure 4F).

The numbers of CD45⁺, Ter119⁺ cells (Figure 5A), and CFU-C (Figure 5B) were also dramatically reduced in the

(C) Irradiated mice were transplanted with 1,000 GFP⁺ fetal liver cells (green lines) or 199,000 GFP⁻ fetal liver cells (red lines) from E14.5 *Sox17*^{GFP/+} donor mice. All mice transplanted with GFP⁺ cells were long-term multilineage reconstituted by donor cells (n = 12; each line represents a single mouse) but none of the mice transplanted with GFP⁻ cells showed detectable reconstitution (n = 11 mice; the black line at 0.3% represents background).

(D) Irradiated recipient mice were transplanted with unfractionated fetal liver, yolk sac, or remaining embryo cells from single E12.5 *Sox17*^{fl/+}, *Sox17*^{GFP/+}, or *Sox17*^{GFP/GFP} embryos. Numbers indicate the fraction of recipient mice that were long-term multilineage reconstituted (>16 weeks) by donor cells.

(E) While control cells usually gave long-term multilineage reconstitution (blue and green lines), we never detected any reconstitution by *Sox17*^{GFP/GFP} cells (red lines).

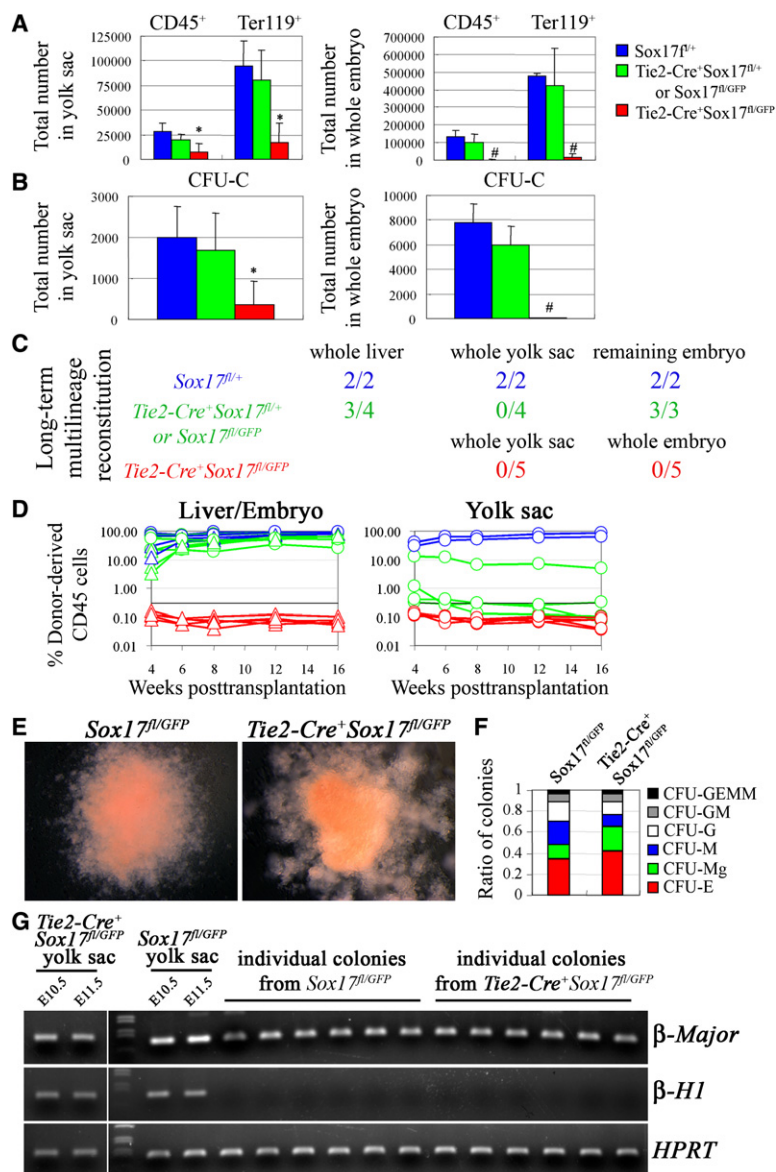


Figure 5. Conditional Deletion of *Sox17* Using *Tie2-Cre* Leads to a Failure to Generate or Maintain HSCs

The numbers of CD45⁺ or Ter119⁺ cells (A) and colony-forming progenitors (CFU-C; B) were dramatically reduced in the yolk sac and embryo of E11.5 *Tie2-Cre⁺Sox17^{fl/GFP}* mice as compared to littermate controls (*, $p < 0.05$; #, $p < 0.01$; four independent experiments).

(C) Irradiated wild-type mice were transplanted with unfractionated fetal liver, yolk sac, or remaining embryo cells from single E12.5 *Tie2-Cre⁺Sox17^{fl/GFP}* embryos or littermate controls. The livers of *Tie2-Cre⁺Sox17^{fl/GFP}* embryos were so hypocellular and pale that the entire embryos were dissociated and transplanted, rather than just fetal liver. Numbers indicate the fraction of recipient mice that were long-term multilineage reconstituted by donor cells.

(D) While control cells usually gave long-term multilineage reconstitution (blue and green lines), we never detected any reconstitution above background (0.3%) from *Tie2-Cre⁺Sox17^{fl/GFP}* cells (red lines).

(E) CFU-GEMM colonies from E11.5 *Sox17^{fl/GFP}* and *Tie2-Cre⁺Sox17^{fl/GFP}* yolk sac were similar in size and appearance.

(F) *Sox17* deletion also did not affect the proportions of colony types, though the absolute number of all colonies was reduced (three independent experiments).

(G) E11.5 *Tie2-Cre⁺Sox17^{fl/GFP}* and *Sox17^{fl/GFP}* yolk sac cells expressed embryonic (β -H1) and adult (β -major) hemoglobin in vivo. In culture, all colonies from *Sox17^{fl/GFP}* yolk sac (19/19) and *Tie2-Cre⁺Sox17^{fl/GFP}* yolk sac (20/20) expressed adult hemoglobin (β -major). PCR on genomic DNA from individual colonies demonstrated that 95% of *Tie2-Cre⁺Sox17^{fl/GFP}* colonies (19/20) had completely recombined *Sox17* (data not shown).

yolk sac and embryo of E11.5 *Tie2-Cre⁺Sox17^{fl/GFP}* mice as compared to controls. As in germline knockouts, these defects were most severe intraembryonically. *Tie2-Cre⁺Sox17^{fl/GFP}* embryos also lacked any reconstituting activity when yolk sac or embryo cells were transplanted into irradiated mice, in contrast to littermate controls (Figure 5C). *Sox17* is thus required in endothelial/hematopoietic cells for the generation or maintenance of definitive HSCs.

***Sox17*-Deficient Progenitors Can Differentiate Normally and Form Definitive Erythrocytes**

To test whether *Sox17*-deficient hematopoietic progenitors were blocked in their ability to differentiate or to transition from primitive to definitive hematopoiesis, we examined the rare colonies that arose in culture from E11.5

Tie2-Cre⁺Sox17^{fl/GFP} yolk sac cells. Although the number of colonies was significantly reduced as compared to littermate controls (Figure 5B), the types of colonies that formed, the size of colonies, their appearance, and their composition did not differ between *Tie2-Cre⁺Sox17^{fl/GFP}* mice and controls (Figures 5E and 5F). Flow-cytometric analysis revealed normal numbers of Ter119⁺ erythrocytes and Mac-1⁺ myeloid cells within colonies from *Tie2-Cre⁺Sox17^{fl/GFP}* mice (data not shown). RT-PCR on freshly dissected, uncultured yolk sac cells revealed both embryonic (β -H1) and adult (β -major) hemoglobin expression from wild-type and *Tie2-Cre⁺Sox17^{fl/GFP}* yolk sac cells in vivo (Figure 5G). All colonies from *Tie2-Cre⁺Sox17^{fl/GFP}* mice and controls expressed adult but not embryonic hemoglobin (Figure 5G). Thus, hematopoietic progenitors are greatly reduced in number in *Tie2-Cre⁺Sox17^{fl/GFP}*

mice but appear to differentiate normally, consistent with a defect in HSC formation or maintenance.

Sox17 Is Cell-Autonomously Required for the Maintenance of Definitive HSCs

To test whether Sox17 is required for the ongoing maintenance of HSCs, or just for their initial formation, we generated *Mx-1-Cre⁺Sox17^{fl/GFP}* mice. We treated newborn *Mx-1-Cre⁺Sox17^{fl/GFP}* mice and littermate controls (that included both *Mx-1-Cre⁻Sox17^{fl/GFP}* and *Mx-1-Cre⁺Sox17^{fl/+}* mice) with three injections of plpC on days 2, 4, and 6 after birth to induce Cre expression (Hock et al., 2004b). This led to the death of all *Mx-1-Cre⁺Sox17^{fl/GFP}* mice by 14 days after birth, while all littermate controls survived (Figure 6A). In separate experiments, neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice and littermate controls were sacrificed 4–5 days after ending plpC treatment to test whether there was any effect on hematopoiesis. *Mx-1-Cre⁺Sox17^{fl/GFP}* mice all exhibited significant ($p < 0.05$) reductions relative to littermate controls in bone marrow cellularity, spleen cellularity, numbers of Flk2⁺Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs, and numbers of CFU-C in the bone marrow and spleen (Figures 6B–6D). These mice also had severely reduced thymus cellularity and significantly reduced white blood cell and platelet counts (data not shown). While CFU-C were substantially depleted after Sox17 deletion, the types of colonies they formed and the appearance and composition of the colonies did not differ from control cells (data not shown). The depletion of Flk2⁺Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs after Sox17 deletion indicates that Sox17 is required for the maintenance of HSCs.

To further test if Sox17 is autonomously required for the maintenance of HSCs, whole bone marrow cells were transplanted from plpC-treated neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice or littermate controls into irradiated wild-type recipients along with wild-type whole bone marrow cells. Bone marrow cells taken from *Mx-1-Cre⁺Sox17^{fl/GFP}* mice 4–5 days after plpC treatment initially gave low levels of multilineage reconstitution, but multilineage reconstitution could no longer be detected by 6 weeks after transplantation (Figure 6E). In contrast, the same dose of bone marrow cells from littermate controls gave high levels of multilineage reconstitution in all recipients (Figure 6E). By performing PCR on genomic DNA extracted from individual CFU-GEMM or CFU-GM colonies that arose from the bone marrow of the donor mice used in these experiments we determined that 81% of early hematopoietic progenitors from the neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice had deleted Sox17 (Figure S4). The failure of Sox17-deficient bone marrow cells to long-term multilineage reconstitute wild-type recipients indicates that Sox17 is autonomously required for the maintenance of neonatal HSCs.

We also tested whether fetal HSCs require Sox17 for their maintenance in a wild-type environment in a way that is independent of their ability to home to the bone marrow after transplantation. We transplanted 200,000

unfractionated fetal liver cells from E14.5 *Sox17^{fl/+}* or *Mx-1-Cre⁺Sox17^{fl/GFP}* embryos into irradiated wild-type adult recipients along with a radioprotective dose of wild-type bone marrow cells. plpC was administered to all recipient mice 5, 7, and 9 days after transplantation; then, the peripheral blood was analyzed 4 weeks after transplantation. High levels of multilineage reconstitution were observed from the control (*Sox17^{fl/+}*) donor cells in all eight recipient mice: $80\% \pm 9\%$ of all CD45⁺ cells were donor derived in these mice 6 weeks after transplantation (Figure S5). In contrast, none of seven recipient mice transplanted with *Mx-1-Cre⁺Sox17^{fl/GFP}* cells were stably multilineage reconstituted: only $5\% \pm 10\%$ of all CD45⁺ cells were donor derived 6 weeks after transplantation (Figure S5). Deletion of Sox17 from fetal HSCs that have already engrafted in a wild-type environment leads to a near complete loss of reconstituting potential, indicating that Sox17 is autonomously required for the maintenance of fetal HSCs.

Since Sox17 expression is extinguished in HSCs around 4 weeks after birth (Figure 3B) we also administered plpC to 6-week-old mice to test whether adult HSCs are no longer Sox17 dependent. None of eight *Mx-1-Cre⁺Sox17^{fl/GFP}* mice died, though one of twenty-eight control mice died (Figure 6A). In separate experiments, *Mx-1-Cre⁺Sox17^{fl/GFP}* mice and littermate controls were sacrificed 1 week after ending plpC. We observed no difference between *Mx-1-Cre⁺Sox17^{fl/GFP}* mice and littermate controls in bone marrow cellularity, spleen cellularity (Figure 6B), number of Flk2⁺Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs in the bone marrow (Figure 6C), numbers of CFU-C in the bone marrow (Fig. 6D), or ability of bone marrow cells to reconstitute irradiated mice (Figure 6E). We also treated adult *Mx-1-Cre⁺Sox17^{fl/GFP}* mice and littermate controls (after plpC treatment) with cyclophosphamide/G-CSF to mobilize HSCs but again detected no effect of Sox17 deficiency on bone marrow cellularity, spleen cellularity, HSC frequency, or HSC cell cycle status (Figure S2). By performing PCR on genomic DNA extracted from colonies that arose in culture from sorted Flk2⁺Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs we confirmed that 96% of the HSCs from adult *Mx-1-Cre⁺Sox17^{fl/GFP}* mice had deleted Sox17 (Figure S4). Sox17 is no longer required for adult HSC maintenance, proliferation, mobilization, or hematopoiesis.

Sox17 Deletion Leads to the Death of Neonatal HSCs

To further investigate the mechanism by which Sox17 deletion leads to the loss of HSCs, we examined Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs isolated from the bone marrow of neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice or littermate controls 5 days after plpC treatment. Sox17 deletion had no immediate effect on the cell cycle distribution of HSCs (Figure 7A); however, Sox17 deletion did significantly ($p < 0.05$) increase the rate of cell death among HSCs (Figure 7B).

To begin to address the molecular mechanisms by which Sox17 promotes the maintenance of HSCs, we

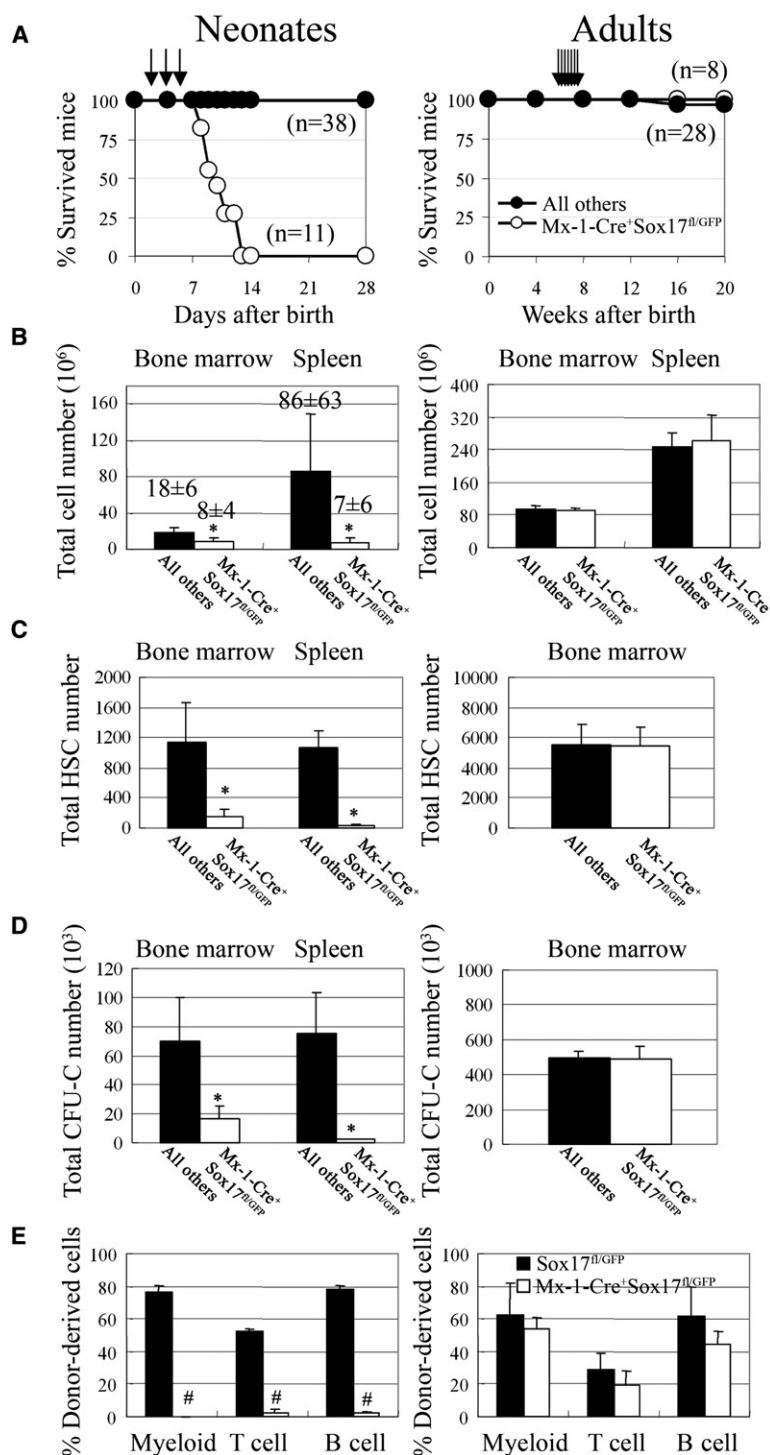


Figure 6. Sox17 Is Autonomously Required for the Maintenance of Neonatal But Not Adult HSCs

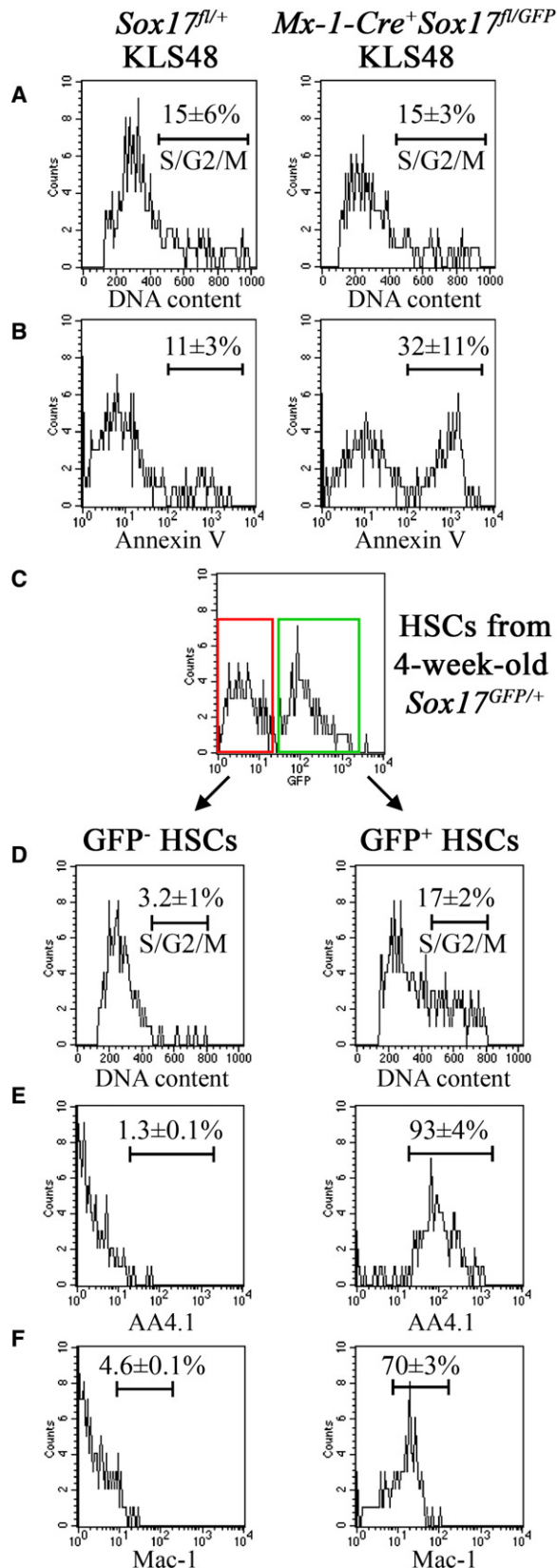
(A) *Mx-1-Cre⁺Sox17^{fl/GFP}* mice and littermate controls were administered plpC 2, 4, and 6 days after birth (arrows). All *Mx-1-Cre⁺Sox17^{fl/GFP}* mice died by 14 days after birth, but no littermate controls died. Adult *Mx-1-Cre⁺Sox17^{fl/GFP}* mice that were administered seven doses of plpC over a 14 day period beginning 6 weeks after birth all survived.

(B–C) Four to five days after ending plpC treatment in neonates and seven days after ending plpC treatment in adults, bone marrow cellularities in the tibias and femurs and spleen cellularities were significantly (*, $p < 0.05$) reduced in neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice ($n = 5–7$) as compared to littermate controls ($n = 4–6$) but were not affected in adult *Mx-1-Cre⁺Sox17^{fl/GFP}* mice ($n = 4–5$) as compared to littermate controls ($n = 4–5$). The absolute numbers of Flk2⁺Sca-1⁺Lineage[−]c-kit⁺CD48[−] HSCs (C) and colony-forming progenitors (CFU-C; D) in the bone marrow (from tibias and femurs) and spleen were also significantly ($p < 0.05$) reduced in the same neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice but were not affected in the adult *Mx-1-Cre⁺Sox17^{fl/GFP}* mice.

(E) Bone marrow cells from plpC-treated neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice (CD45.2⁺) failed to give long-term multilineage reconstitution in irradiated CD45.1⁺ wild-type recipients ($n = 5$), in contrast to the same dose of cells from littermates or adult mice ($n = 4$ or 5; differences in myeloid, B, and T cell chimerism were highly statistically significant: #, $p < 0.001$; 6 week time point is shown). In each case, 200,000 donor bone marrow cells were competed against 200,000 recipient bone marrow cells.

compared the expression of candidate genes by hematopoietic progenitors isolated from *Sox17*-deficient or control mice. We first tested whether *Sox17* was required for the expression of *Runx1*, *Scl*, *Gata-2*, or *Lmo2*. However, all of these genes were expressed at approximately wild-type levels in both CD45⁺c-kit⁺ progenitors and CD45⁺c-kit[−] hematopoietic cells from E11.5 *Tie2-*

Cre⁺Sox17^{fl/GFP} embryos and yolk sacs (Figure S6). *Sox17* was therefore not required for the expression of genes involved in the formation of HSCs, nor for the expression of other critical regulators of HSC function including *c-Myb* and *Gfi-1* (Figure S6). In striking contrast, *Dickkopf1* (*Dkk1*) expression was dramatically elevated, by 142-fold in CD45⁺c-kit⁺ progenitors from E11.5 *Tie2-Cre⁺Sox17^{fl/GFP}*



embryos (Figure S6). *Dkk1* expression was also elevated 3.4-fold in the same population from yolk sac. Since *Dkk1* is a powerful negative regulator of Wnt pathway activation (Glinka et al., 1998) these data raise the possibility that *Sox17* is required to maintain Wnt signaling in HSCs.

To further explore this possibility we examined gene expression in c-kit⁺Lineage⁻CD48⁻ hematopoietic progenitors from the bone marrow of neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice or littermate controls 3 days after plpC treatment. Out of 15 genes examined, including those listed above as well as *Bmi-1*, *Etv6*, *Rae28*, *Meis1*, and *Cpb*, the only gene that showed more than a 2-fold change in expression after *Sox17* deletion was *Dkk1* (3.3 ± 0.4 -fold increased expression). Thus *Dkk1* expression was elevated in every context in which *Sox17* deficiency led to a loss of HSCs. These data raise the possibility that *Sox17* may promote the maintenance of fetal and neonatal HSCs by promoting Wnt signaling.

Sox17 Expression Is Associated with the Maintenance of Fetal HSC Properties

To test if the decline in *Sox17* expression in wild-type postnatal HSCs is associated with the acquisition of adult properties we examined 4-week-old *Sox17^{GFP/+}* mice. At this time HSCs are making the transition from a rapidly proliferating fetal phenotype to a quiescent adult phenotype (Bowie et al., 2006), and approximately 50% of Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs express *Sox17* (Figure 3B). To test whether the loss of *Sox17* expression is associated with the loss of the rapidly proliferating phenotype, we compared the cell-cycle status of GFP⁺ and GFP⁻ Sca-1⁺Lineage⁻c-kit⁺CD48⁻ cells from *Sox17^{GFP/+}* mice. While GFP⁺ Sca-1⁺Lineage⁻c-kit⁺CD48⁻ cells remained rapidly proliferating, with 17% ± 2% of cells in S/G2/M phase of the cell cycle, GFP⁻ Sca-1⁺Lineage⁻c-kit⁺CD48⁻ cells were dividing significantly more slowly, with only 3% ± 1% of cells in S/G2/M (Figure 7D). This demonstrates that the loss of *Sox17* expression is associated with the acquisition of a more slowly dividing adult phenotype.

Figure 7. Sox17 Deletion from Neonatal HSCs Induces Cell Death, and the Postnatal Decline in Sox17 Expression by Wild-Type HSCs Is Associated with a Transition to an Adult Phenotype

(A and B) Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs isolated from the bone marrow of neonatal *Mx-1-Cre⁺Sox17^{fl/GFP}* mice 5 days after plpC treatment exhibited normal cell-cycle distribution relative to littermate controls (A) but a 3-fold increased frequency of cells undergoing cell death (Annexin V⁺; B).

(C–F) GFP⁺ (*Sox17*-expressing) and GFP⁻ (*Sox17*-non-expressing) Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs were isolated from the bone marrow of 3.5 to 4 week-old *Sox17^{GFP/+}* mice. GFP⁻ Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs were dividing less rapidly (D), and failed to express AA4.1 (E) or Mac-1 (F), consistent with an adult HSC phenotype. In contrast, GFP⁺ Sca-1⁺Lineage⁻c-kit⁺CD48⁻ HSCs were more rapidly dividing (D), and expressed AA4.1 (E) and Mac-1 (F), consistent with a fetal HSC phenotype.

To test whether the loss of *Sox17* expression is also associated with the acquisition of an adult surface marker phenotype, we compared the expression of AA4.1 and Mac-1 between the GFP⁺ and GFP⁻ fractions of Sca-1⁺Lineage⁻c-kit⁺CD48⁻ cells from *Sox17*^{GFP/+} mice. Mac-1 and AA4.1 are expressed by fetal but not adult HSCs (Morrison and Weissman, 1994; Morrison et al., 1995; Phillips et al., 2000). Note that when sorting Sca-1⁺Lineage⁻c-kit⁺CD48⁻ cells from neonatal mice we did not include Mac-1 in the lineage cocktail. While GFP⁺ Sca-1⁺Lineage⁻c-kit⁺CD48⁻ cells were mainly Mac-1⁺ and AA4.1⁺, GFP⁻ Sca-1⁺Lineage⁻c-kit⁺CD48⁻ cells were mainly Mac-1⁻ and AA4.1⁻ (Figures 7E and 7F). This demonstrates that the loss of *Sox17* expression is associated with the acquisition of an adult surface marker phenotype by HSCs.

DISCUSSION

The discovery that *Sox17* maintains fetal but not adult HSCs fills a critical void in our understanding of HSC regulation. Prior studies identified genes like *Scl*, *Aml-1*/*Runx1*, *Lmo2*, and *Gata-2* that are required for the formation of fetal HSCs (Warren et al., 1994; Okuda et al., 1996; Porcher et al., 1996; Wang et al., 1996; Mikkola et al., 2003; Ichikawa et al., 2004), and genes like *Gfi-1*, *Tel*/*Etv6*, and *Bmi-1* that are required for the maintenance of adult but not fetal HSCs (Park et al., 2003; Hock et al., 2004a; Hock et al., 2004b). However, we are not aware of other genes that are required to maintain fetal HSCs prior to the acquisition of adult HSC properties.

Our data suggest that *Sox17* acts downstream of *Scl*, *Aml-1*/*Runx1*, *Lmo2*, and *Gata-2* to maintain fetal HSCs prior to the acquisition of an adult phenotype. Quantitative RT-PCR on RNA from CD45⁺c-kit⁺ hematopoietic progenitors from E11.5 *Sox17*-deficient embryos showed normal or slightly elevated levels of *Scl*, *Aml-1*/*Runx1*, *Lmo2*, and *Gata-2* transcripts (Figure S6). This demonstrates that *Sox17* is not required for the expression of *Scl*, *Aml-1*/*Runx1*, *Lmo2*, or *Gata-2* in embryonic hematopoietic cells around the time that definitive HSCs are emerging. *Sox17* likely acts downstream of these genes to maintain fetal HSCs after they have formed. Our data do not rule out a role for *Sox17* in the formation of definitive HSCs, but there are no data that directly support such a possibility so far.

Sox17 may be required to maintain fetal and neonatal HSCs by promoting Wnt signaling. Neonatal HSCs underwent cell death within days of *Sox17* deletion (Figure 7B), and *Sox17* deletion dramatically increased *Dkk1* expression in neonatal and embryonic hematopoietic progenitors (Figure S6). *Dkk1* negatively regulates Wnt pathway activation (Glinka et al., 1998), suggesting that *Sox17* deletion may lead to reduced Wnt signaling in HSCs. Consistent with this possibility, *Sox17* can negatively regulate the expression of β -catenin target genes in some contexts (Zorn et al., 1999; Sinner et al., 2004). Moreover, *Wnt3a*-deficient mice (Takada et al., 1994) and *Tcf4*/*Tcf1*-deficient

mice (Gregorieff et al., 2004) have posterior patterning defects that are similar to the posterior patterning defect we observed in *Sox17* deficient mice (Figure 1). Wnt pathway activation has been implicated in the regulation of adult HSC self renewal (Reya et al., 2003; Willert et al., 2003), though it may not be necessary (Cobas et al., 2004), and it is unknown whether Wnt signaling is important for fetal HSC maintenance. Additional studies will be required to test whether *Sox17* promotes the maintenance of fetal/neonatal HSCs by promoting Wnt signaling. We also do not know whether overexpression of *Sox17* in adult HSCs would be sufficient to induce a fetal phenotype.

The identification of *Sox17* as a critical regulator of fetal/neonatal HSCs demonstrates that the transcriptional programs that maintain HSCs change over time. This implies that the regulation of stem cell identity may change with developmental time, perhaps in concert with functional changes. The discovery that *Sox17* is autonomously required for the maintenance of fetal and neonatal but not adult HSCs identifies a new stage-specific mechanism for the maintenance of HSCs that fills a gap between previously identified mechanisms for the embryonic formation of HSCs and the maintenance of adult HSCs.

EXPERIMENTAL PROCEDURES

See Supplemental Data for details regarding the generation of *Sox17* mutant mice.

Flow Cytometry and Isolation of HSCs

Fetal liver or bone marrow cells (flushed from tibias and femurs of adult mice) were trituated with Hank's Buffered Salt Solution without calcium or magnesium, supplemented with 2% heat-inactivated calf serum (Gibco, Grand Island, NY; HBSS⁺) and filtered through nylon screen (45 μ m, Sefar America; Kansas City, MO) to obtain a single cell suspension. To examine the expression of GFP in fetal liver HSCs (Sca-1⁺Lin⁻Mac-1⁺CD48⁻) and adult bone marrow HSCs (Sca-1⁺Lin⁻c-kit⁺CD48⁻), fetal liver cells and bone marrow cells were stained as previously described (Kiel et al., 2005b; Kim et al., 2006). Whole fetal liver cells were incubated with unconjugated monoclonal antibodies to lineage markers including B220 (6B2), CD3 (KT31.1), CD5 (53-7.3), CD8 (53-6.7), Gr-1 (8C5), and Ter119 (Ter-119). Pelleted cells were resuspended in anti-rat IgG specific F(ab)₂ conjugated to phycoerythrin (PE; Jackson ImmunoResearch; West Grove, PA). Cells were then stained with directly conjugated antibodies to Mac-1 (M1/70-allophycocyanin (APC)), Sca-1 (Ly6A/E-biotin), CD48 (HM48-1-PE), and CD4 (GK1.5-PE), followed by staining with streptavidin conjugated to APC-Cy7 (PharRed; PR) (BD Biosciences; San Jose, CA). Adult whole bone marrow cells were stained in the same manner as whole fetal liver cells except that different fluorochromes were used for Mac-1 (M1/70-PE) and Sca-1 (Ly6A/E-APC), and c-kit (2B8-biotin) was also included as a marker.

To analyze the expression of GFP in E10.5 or E11.5 embryos, embryos and yolk sacs were separated carefully and digested with collagenase type I (1 mg/ml) in the presence of 10% FBS at 37°C for 1.5 hr as previously described (Gekas et al., 2005). Isolated cells were stained with unconjugated anti-CD144 (clone 11D4.1; BD Biosciences), which was detected by anti-rat IgG-APC (Jackson ImmunoResearch), anti-CD45-biotin (clone 30-F11; eBioscience, San Diego, CA), and Ter119-PE.

Cells were resuspended in 2 μ g/ml 7-AAD (Molecular Probes; Eugene, OR) to discriminate live from dead cells. All flow cytometry

was performed on a FACSVantage SE-dual laser, three-line flow cytometer (Becton-Dickinson).

Long-Term Competitive Reconstitution Assays

Recipients in reconstitution assays were adult C57Bl/Ka-CD45.1:Thy-1.2 mice. Adult recipient mice were irradiated with an Orthovoltage x-ray source delivering approximately 300 rads/min in two doses of 550–570 rad each, delivered at least 2 hr apart. Transplanted cells were injected into the retro-orbital venous sinus of individual lethally irradiated recipients along with 200,000 recipient bone marrow cells for radioprotection. For at least 16 weeks after transplantation, blood was obtained from the tail veins of recipient mice, subjected to ammonium-chloride/potassium bicarbonate red cell lysis, and stained with directly conjugated antibodies to CD45.2 (104), B220 (6B2), Mac-1 (M1/70), CD3 (KT31.1) and Gr-1 (8C5) to monitor donor cell engraftment.

Methylcellulose Culture

Cells were plated in wells of 96-well plates (Corning; Corning, NY) containing 100 μ l of 1.0% MethoCult GFM3434 (Stem Cell Technologies; Vancouver, Canada).

Supplemental Data

Supplemental Data include six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://www.cell.com/cgi/content/full/130/3/470/DC1/>.

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